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United Kingdom

Patents ADP number *(if you know it)* 7983207001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — ENZYME INHIBITION

5. Name of your agent *(if you have one)*

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ENZYME INHIBITION

The present invention relates to enzyme inhibition, and in particular to the inhibition of an enzyme that we call asparaginyl endopeptidase which we have found is involved in processing antigens, particularly microbial antigens, by the immune system.

The immune response to protein antigens involves a large number of individual gene products, and new ones are still being discovered. In some cases the gene products were known *per se*, but not known to be involved in the immune response.

An immune response may be raised towards foreign antigens, for example, antigens associated with microorganisms, or the immune response may be caused by a response to a self antigen (autoimmunity). In either case, there exists the need for methods and means for modulating the immune system and how it responds to these foreign and self antigens.

Because proteins must be proteolytically processed (ie partially degraded) before the T cells of the immune system can respond, one set of proteins of importance in an immune response are proteolytic enzymes (proteases). It is typically proteolysed proteins (ie protein fragments) which are presented by antigen presenting cells on MHC Class I or Class II molecules.

Several different aspartic and cysteine proteases are thought to be involved in invariant chain and antigen processing (see, for example, Fineschi & Miller (1997) *Trends Biochem. Sci.* **22**, 377-382; and Chapman (1998)

Curr. Op. Immunol. **10**, 93-102). For example, there is now good evidence that cathepsin S plays a role in the final stages of invariant chain processing (see, for example, Riese *et al* (1996) *Immunity* **4**, 357-366; and Villadangos *et al* (1997) *J. Exp. Med.* **186**, 549-560).

We have now found, surprisingly, a further protease which is involved in processing protein antigens. The enzyme has asparaginyl endopeptidase (AEP) activity and until the work described in this patent application, was not known to be involved in the immune response.

An enzyme with AEP activity was first detected in legumes and was called "legumain". Enzymes with AEP activity have also been detected in the blood fluke *Schistosoma mansoni* where it is involved in degrading haemoglobin (haemoglobinase) and, more recently, in mammals from which the cDNA has also been cloned (Chen *et al* (1997) *J. Biol. Chem.* **272**, 8090-8096).

A first aspect of the invention provides a method of modulating the immune response in a patient in need of such modulation, the method comprising administering to the patient an effective amount of an inhibitor of asparaginyl endopeptidase.

We have found that an enzyme with AEP activity is present in the lysosomes of B cells and is involved in degrading certain proteins. It is fragments of a protein, produced following proteolytic degradation, which are loaded onto and presented by Class II MHC molecules. Inhibition of AEP activity has been shown to interfere with the degradation of certain asparagine-containing proteins and to substantially inhibit the loading and

presentation of peptides on Class II MHC molecule-containing cells (and to substantially inhibit T cell activation).

An enzyme with AEP activity is readily able to cleave the substrate Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide to release a fluorescent product, where Z is benzyloxy carbonyl.

By an "inhibitor of asparaginyl endopeptidase" we include any suitable inhibitor. The inhibitor may be a competitive inhibitor or it may be a non-competitive inhibitor. AEP is a cysteine protease and so inhibitors which chemically react with the active cysteine residue are suitable and are, typically, irreversible inhibitors. Other amino acid residues, such as histidine, may be present at the active site and inhibitors which react with any active site residue are suitable.

In one embodiment of the invention the AEP inhibitor is a competitive inhibitor. Typically, the competitive inhibitor is a peptide comprising an asparagine-containing peptide. Suitably, the peptide is a peptide comprising a known AEP cleavage site but which, because of its affinity for AEP can compete for another AEP cleavage site and substantially prevent cleavage at the said other cleavage site if present in sufficient concentration. Known cleavage sites for AEP in tetanus toxin are described in Chen *et al* (1997) *J. Biol. Chem.* **272**, 8090-8098.

In a preferred embodiment of the invention the competitive inhibitor has the general structure $(X_1)_p\ N(X_2)_q$ wherein X_1 and X_2 are amino acid residues, N is an asparagine residue, p is 3 to 6 and q is 1 to 3.

Suitably, the competitive inhibitor is a peptide which comprises the peptide sequence Ala-Glu-Asn-Lys or, less preferably, Lys-Asn-Asn-Glu. Preferably, the competitive inhibitor is an N and C-terminal blocked peptide Ala-Glu-Asn-LysNH (AENK) or, less preferably, Lys-Asn-Asn-Glu-NH (KNNE).

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using

ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

In a further embodiment, the inhibitor may be a non-competitive or irreversible inhibitor. AEP activity can be blocked by high concentrations of inhibitors which block all cysteine proteases and some serine proteases. However, it is particularly preferred that the inhibitors are selective for AEP.

Generally, irreversible AEP inhibitors may be any of peptide aldehydes, peptide chloromethyl ketones, peptide fluoromethyl ketones, peptide

diazomethanes or peptide vinyl sulphones, peptide (acyloxy)methanes, peptide N, O diacyl hydroxamates, or peptides which bind AEP and have a reactive group which will react with the active cysteine residue or other residue at the active site.

Suitably, the peptide sequence has the C-terminal residue as an asparagine residue to which is attached the group which reacts with the active site cysteine. The position of the asparagine residue in the peptide inhibitors (ie at the C terminus before the active group) is analogous to the P1 position at a cleavage site.

Thus, in general, an AEP inhibitor has the structure Bl-$(X)_n$-Asn-Q where Bl is a suitable N-terminal blocking group including, for example, acetyl or benzyloxycarbonyl; X is an amino acid residue; n is between 1 and 100, preferably between 1 and 50, more preferably between 1 and 10 and typically 2 to 6; Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine or other active site residue of AEP and, conveniently, forming a covalent complex thereby eliminating catalytic activity. It is preferred if the amino acid residues in the peptide X_n-Asn are in the L configuration and it is particularly preferred that, whether or not the X_n amino acid residues are in the L-configuration, that Asn is in the L-configuration. The amino acid residues X may be any naturally occurring amino acid residues or they may be residues which have non-natural side chains.

The peptide sequence before the C terminal asparagine and active group may be any suitable peptide sequence, but it will be appreciated that it is the sequence at a known AEP cleavage site.

Typically, peptide aldehyde inhibitors have the structure:

Acetyl (or other blocking group)-X_n-Asparaginal

where X is an amino acid residue and n is between 1 and 100, preferably between 1 and 50, more preferably between 1 and 10 and typically 2 to 6.

A suitable peptide aldehyde includes acetyl-alanyl-glutamyl-asparaginal.

Elastatinal blocks AEP activity; however a more specific variant, in which the C terminal amino residue is replaced by asparagine, may be useful.

Figure 4 shows the structures of some useful inhibitors.

Typically, peptide chloromethylketone inhibitors have the structure.

Acetyl (or other blocking group)-X_n-asparaginyl-chloromethyl ketone where X and n are as above.

A suitable peptide includes acetyl-alanyl-glutamyl-asparaginyl-chloromethylketone and acetyl-tyrosyl-valyl-alanyl-asparaginyl-chloromethylketone (analogous to ICE protease inhibitor YVAD-cmk); see Figure 4 for further details.

Typically, peptide fluoromethylketone inhibitors have analogous structures to peptide chloromethylketone inhibitors except for the replacement of a chloro group with a fluoro group.

Typically, peptide diazomethane inhibitors have the structure

$$Bl\text{-}(X)_n\text{-diazomethane}$$

where Bl is any suitable blocking group including acetyl or benzyloxycarbonyl and X and n are as above. A diazomethane on the C-terminus of a peptide has a general structure $R\text{-}C(O)CHN_2$ where R represents the peptide.

A suitable peptide includes Bl-alanyl-asparaginyl-diazomethane or Bl-alanyl-glutamyl-asparaginyl-diazomethane.

Typically, peptide vinyl sulphone inhibitors have the structure

$$Bl\text{-}(X)_n\text{-vinyl sulphone-R}$$

where Bl is an N-terminal blocking group such as acetyl or benzyloxycarbonyl, X and n are as above, and R is any suitable alkyl or aryl terminating group and includes C_1 to C_{10} alkyl, phenyl, benzyl, naphthyl and the like.

A suitable peptide includes morpholinurea-leucyl-asparaginyl-vinyl sulphone-phenyl or morpholinurea-alanyl-glutamyl-asparaginyl-vinyl sulphone-phenyl.

Other suitable peptides include peptidyl(acyloxy) methanes and peptidyl N,O-diacylhydroxamates.

It will be appreciated from the foregoing that AEP inhibitors may be designed based on other cysteine protease inhibitors such as E-64 (1-trans-epoxysuccinyl-leucylamide (4-guanido)-butane), Leupeptin (acetyl-leucyl-leucyl-arginal), Antipain ([(S)-1-carboxy-2-phenyl]-carbamoyl-Arg-Val-arginal), Elastinal (N-[(S)-1-carboxy-isopentyl)-carbamoyl-α-(2-iminohexahydro-4(S)-pyrimidyl]-L-glycyl-L-glutaminyl-L-alaninal), TLCK (tosyllysylchloromethylketone) and TPCK (tosylphenylalanylchloromethylketone), for example by introduction of an asparaginyl residue at an appropriate place in place of an existing amino acid residue.

Vinyl sulphone inhibitors of cysteine proteases are described in Palmer *et al* (1995) *J. Med. Chem.* **38**, 3193-3196. Peptide aldehyde inhibitors of cysteine proteases are described in Thomson *Methods Enzymol.* **46**, 220 - and Vinitsky *et al* (1994) *J. Biol. Chem.* **269**, 29860-29866 639-648. Peptide diazomethanes as inhibitors of cysteine proteases are described in Shaw (1994) *Methods Enzymol.* **244**, 649-656 and Shaw & green (1981) *Methods Enzymol.* **80**, 820-826. Peptide (aryloxy) methanes as inhibitors of cysteine proteases are described in Krantz (1994) *Methods Enzymol.* **244**, 656-671. Peptide N,O-diarylhydroxamates as inhibitors of cysteine proteases are described in Brömme & Demuth (1994) *Methods Enzymol.* **244**, 671-685. Peptide chloromethyl ketones as inhibitors of cysteine proteases are described in Williams & Mann (1993) *Methods Enzymol.* **222**, 503-513. Thus, methods for synthesising suitable inhibitors are well known to the person skilled in the art.

It is possible that, by analogy with other cysteine proteases, natural inhibitors of AEP exist, for example peptide inhibitors found in mammalian cells, or inhibitors found in microorganisms. The inhibitors for use in the invention include these inhibitors.

It is preferred that the AEP inhibitor is selective for AEP. By "selective" we mean that the inhibitor is at least ten times more potent for AEP than for another cysteine protease that does not have asparaginyl endopeptidase activity.

More preferably, the AEP inhibitor is at least 100 times more potent for AEP than for another cysteine protease inhibitor, still more preferably at least 1000 times more potent and most preferably at least 10 000 times more potent.

The AEP inhibitor, suitably, following administration to the patient, is able to contact and inhibit AEP at a suitable site in the patient. Although it may be desirable for the AEP inhibitor to be cell permeant, it is believed that non-permeant AEP inhibitors may be useful since they may be taken into an appropriate cell by endocytosis.

An "effective amount" of the AEP inhibitor is an amount effective to modulate the immune response in the patient to a clinically useful extent.

AEP is believed to be involved in processing of protein antigens which are destined to be loaded into and presented by Class II MHC molecules. In a preferred embodiment of the invention the patient to be treated has or is at risk of a disease which involves MHC Class II molecules. Although we

are not bound by any theory concerning the invention, we believe that AEP may play an important role in autoimmune disease because it may recognise and cleave at sites which are normally hidden by glycosylation at asparagine residues. Glycosylation at asparagine residues is common in mammalian proteins and glycosylated asparagine residues are not cleaved by AEP. An abnormal reduction in asparagine glycosylation of a self protein may lead to it being susceptible to AEP cleavage at these cryptic sites and, therefore, the peptide produced from the abnormally glycosylated protein may be susceptible to loading into and presentation to the immune system by Class II MHC molecules. Bacterial proteins, such as tetanus toxin, are typically not asparagine glycosylated and so, if they contain appropriate asparagine residues, are susceptible to degradation by AEP and presentation on Class II MHC molecules in any case.

Preferably, the diseases are autoimmune diseases and it is particularly preferred if the patient to be treated has or is at risk of an autoimmune disease such as rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, Hashimoto's thyroiditis, coeliac disease, myasthenia gravis, pemphigus vulgaris, systemic lupus erythromatosus and Grave's disease.

The aforementioned inhibitors for use in the invention or a formulation thereof may be administered by any conventional method including oral, parenteral (eg subcutaneous or intramuscular) injection topical and the like. The treatment may consist of a single dose or a plurality of doses over a period of time.

Whilst it is possible for an inhibitor for use in the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

It is particularly preferred if the AEP inhibitor is administered at or near to the site of the disease. For example, in the treatment of rheumatoid arthritis it is desirable for the AEP inhibitor to be administered at or close to the joints affected. In this case, the AEP inhibitor may be administered by topical application.

A further aspect of the invention provides the use of an inhibitor of asparaginyl endopeptidase in the manufacture of a medicament for modulating the immune response in a patient in need of such modulation.

A still further aspect of the invention provides use of an inhibitor of asparaginyl endopeptidase for modulating the immune response in a patient in need of such modulation.

Yet still further aspects of the invention provide an inhibitor of asparaginyl endopeptidase for use in medicine and a pharmaceutical composition comprising an inhibitor of AEP and a pharmaceutically acceptable carrier.

The invention also includes a method of reducing the processing of a protein antigen by a MHC Class II molecule by a cell, the method

comprising contacting the cell with an inhibitor of asparaginyl endopeptidase.

In one embodiment of the invention, a T cell causing a disease, such as one of the autoimmune diseases listed above, is transfected with a pool of cDNAs (or is contacted with proteins expressed from a pool of cDNAs) wherein the pool of cDNAs is believed to contain a cDNA encoding a protein which activated the T cell. The cDNA which encodes a protein which activates the T cell is selected and encodes a putative autoimmune protein. The assay may be carried out in the presence or absence of AEP inhibition in order to determine the involvement of AEP processing in the disease and also to determine any cryptic Asn glycosylation sites which may be important in the self-antigen immune response.

A further aspect of the invention provides a method of identifying a compound for modulating Class II MHC antigen processing the method comprising contacting a test compound with asparaginyl endopeptidase and selecting a compound which reduces its activity.

This method (or screening assay) of the invention is suitable carried out in a format which allows for many test compounds to be screened simultaneously such as in a 96-well plate format. The activity of AEP is conveniently measured using any suitable substrate but it is preferred if the substrate is one which, upon cleavage by AEP, gives rise to a readily-detectable product. For example, the product may be coloured or fluorescent or detectable in some other way. It is particularly preferred if the product is fluorescent; a preferred substrate is Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide.

The test compound is preferably a compound from a library of test compounds. The compound may be one which has been made by synthetic chemistry methods (such as combinatorial chemistry methods) or it may be a naturally occurring compound. Without prejudice to the type of compounds which can usefully be screened (which include any suitable compounds), it is believed that many inhibitors of AEP will have a peptide or peptide-like structure. Thus, it is preferred if the test compound has a peptide or peptide-like structure.

It will be appreciated that the method or screening assay may also include a step for eliminating non-specific inhibitory compounds or for positively selecting those inhibitors which inhibit AEP but do not substantially inhibit other cysteine proteases which do not have AEP activity.

In a preferred embodiment of the invention, any test compounds which are identified as being capable of reducing the activity of AEP are selected for a second screening step which involves assessing their capacity to influence Class II MHC molecule loading and presentation in a suitable cell.

Thus, peferably the second step is the determination of whether the compound is capable of substantially inhibiting the loading and presentation of peptides on an appropriate Class II MHC molecule-containing cell. A convenient way of determining this is to assess whether the compound is capable of substantially inhibiting T cell activation by an appropriate Class II MHC molecule-containing cell. This may be done as described in the Example.

A further aspect of the invention comprises a non-human transgenic animal wherein a gene encoding asparaginyl endopeptidase has been modified and the animal expresses substantially no asparaginyl endopeptidase from said gene.

By "transgenic" animal we specifically include animals in which all or part of a gene have been "knocked out" or otherwise made substantially incapable of expressing an asparaginyl endopeptidase. Suitable mice can be made using standard methodology involving, for example, genetic manipulation of embryonic stem (ES) cells as is well known in the art. A cDNA encoding AEP is described in Chen *et al* (1997) *J. Biol. Chem.* **272**, 8090-8096, and this information may be used in designing and making the transgenic animals of the invention.

It is particularly preferred if the non-human animal is a mouse, but it may be any suitable non-human animal including rat, rabbit and the like.

In a preferred embodiment of the invention, the non-human transgenic animal which expresses substantially no AEP from an AEP gene comprises a genetic background which predisposes the animal to an autoimmune disease either spontaneously or upon administration of protein antigen. Animals, especially mice, with a suitable genetic background are well known, for example NOD mice, which are a model for diabetes, EAE mice which are a model for allergic encephalomyelitis and CIA mice which are a model for collagen-induced arthritis. Such mice are described or referenced in Cantorna *et al* (1998) *J. Nutr.* **128**, 68-72; Xiao & Link

(1997) *Clin. Immunol. Immunopathol.* **85**, 119-128; and Kumar *et al* (1997) *J. Exp. Med.* **185**, 1725-1733.

Typically, the animal will be transgenic for a human Class II MHC molecule that predisposes to a human autoimmune disease.

In a further preferred embodiment, the non-human transgenic animal which expresses substantially no AEP from an AEP gene is further transgenic for a human Class II MHC molecule and, optionally, further transgenic for CD4. Mice with these further characteristics are described in Altmann *et al* (1995) *J. Exp. Med.* **181**, 867-875; Yamamoto *et al* (1994) *J. Ex. Med.* **180**, 165-171; and Woods *et al* (1994) *J. Exp. Med.* **180**, 173-181.

It will be appreciated that non-human animals which have combinations of characteristics can be made by crossing appropriate animals. Thus, the animals of the preferred embodiments of the invention may be made by crossing suitable animals.

The invention will now be described by reference to the following Examples and Figures wherein

Figure 1: Processing of TTCF by a leupeptin-insensitive cysteine endopeptidase activity. (a) TTCF was digested *in vitro* in the presence of disrupted lysosomes (1-2 μg) in the presence or absence of iodoacetamide (1 mM), leupeptin, E64 or pepstatin (each at 0.1 mg/ml). Digestions were in 0.1M NH_4 acetate at pH 4.5 or Na_2HPO_4, pH 6.0 or 7.0. After 4 hours at 37°C, the reactions were separated by Tris-tricine SDS-PAGE

and stained with Coomassie Blue. (b) Cleavage sites: TTCF digestion products were separated by SDS PAGE and electrophoretically transferred to nitrocellulose membrane. Individual fragments numbered 1-6 were subjected to 5 cycles of Edman degradation. N-terminal sequence obtained is shown in bold following upstream residues from TTCF (tetanus toxin numbering). The N-terminal sequence of His-tagged TTCF was obtained for fragments 1 and 3. (c) Residues Asn 1184 and Glu 1184 in TTCF were mutated to alanine (see Methods) and digestions performed as before. The mutation abolishes cleavage at the mutated site but not at the first site (doublet at 47 kD). In this experiment cleavage of the third site was minimal.

Figure 2: TTCF is processed by an asparaginyl endopeptidase. (a) Chromatography of the TTCF processing activity on Mono-S resin. Fractions eluted by NaCl gradient were incubated with the substrates Z-Ala-Ala-Asn-NHMec or Z-Phe-Arg-NHMec in the presence or absence of E64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane). E64 had no inhibitory effect on the cleavage of Z-Ala-Ala-Asn-NHMec (not shown). (b) Fractions as above were incubated with 10 μg TTCF in the presence or absence of E64 for 4 hours at 37°C prior to SDS-PAGE analysis. (c) Each fraction was analysed by SDS-PAGE and Western blotting. Electrophoretically transferred material was probed with an affinity purified antiserum raised against the peptide KGIGSGKVLKSGPQC from human legumain[1].

Figure 3: Peptide inhibitors of AEP block asparaginyl endopeptidase activity and TTCF processing *in vitro* and *in vivo*. (a) TTCF was digested with purified AEP in the presence or absence of 0.2 mg/ml of the peptides

indicated. Peptides were N and C-terminally blocked by not removing Fmoc or amide groups respectively. (b) Purified AEP or a crude lysosomal protease mixture were incubated with Z-Ala-Ala-Asn-NHMec, Z-Phe-Arg-NHMec (cathepsins L&B) or Z-Val-Val-Ala NHMec (cathepsin S) in the presence of increasing concentrations of AENK or AEQK. Release of 7-amino-4-methyl coumarin was measured after 10, 20 or 30 minutes. (c) Peripheral blood mononuclear cells were isolated from donor A.K. and preincubated for 15 minutes in the presence or absence of 1 mg/ml AENK (■) or AEQK (○) prior to addition of TTCF antigen. At different times the cells were washed, fixed in 0.05% glutaraldehyde and co-cultured with autologous TTCF specific T cell clones.

Figure 4 shows the structure of various AEP inhibitors.

Figure 5 shows the structure of various protease inhibitors which can be converted to AEP inhibitors as described in the text.

Example 1: Asparaginyl endopeptidase is involved in processing and class II MHC presentation of a microbial antigen

Summary

Foreign antigens must be proteolytically processed to allow loading of peptides onto class II MHC molecules. To investigate which proteases might be involved we exposed a domain of the microbial antigen, tetanus toxin (TTCF) to disrupted lysosomes purified from a human B cell line. Surprisingly, the dominant processing activity was not one of the known lysosomal cathepsins but rather an asparagine specific cysteine endopeptidase which we call AEP. This enzyme appears similar if not identical to a mammalian homologue of legumain[1], an asparaginyl endopeptidase found originally in plants and parasites[2,3]. We designed competitive peptide inhibitors of AEP which specifically block its asparaginyl endopeptidase activity and inhibit processing of TTCF *in vitro* and its presentation *in vivo* to T cells. Since N-glycosylation renders asparagine resistant to cleavage by AEP we suggest that this enzyme may represent a further example of the ability of the innate immune system to focus its attention on microbial non-self.

To analyse processing of a foreign antigen without making prior assumptions about which enzymes are involved we exposed a 47 kD domain of the tetanus toxin antigen (TTCF) to disrupted lysosomes isolated from the human B cell line EDR. As shown in Fig 1a, the antigen was fragmented to produce a discrete series of products at pH 4.5 but not at pH 7.0 and minimally at pH 6.0, confirming the lysosomal origin of the protease(s) involved (Fig 1a). Surprisingly, we could not

inhibit lysosomal digestion of TTCF with leupeptin or E64(trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), broad spectrum inhibitors of lysosomal cysteine proteases or with pepstatin, an inhibitor of the aspartic acid cathepsins E and D (Fig 1a). LHVS, a more specific inhibitor of cathepsin S^4 was also without effect (not shown). Nor could we reproduce the lysosomal TTCF digestion pattern or indeed generate any of these fragments using purified cathepsins L, S, B, D or E , all of which have been implicated in the class II MHC antigen processing pathway (reviewed in refs 5 & 6). Further inhibitor studies revealed however that the activity was sensitive to iodoacetamide (Fig 1a), to N-ethyl maleimide and to high concentrations of the diazomethane, Z-Phe-Phe-CHN2 (not shown) indicating that one or more cysteine protease(s) were nonetheless involved.

We sequenced each major TTCF digestion products to gain more information on this processing activity. All visible fragments arose from 3 cleavages in the TTCF protein and each occurred after an asparagine residue (Fig 1b). Mutation of asparagine 1184 and glutamic acid 1185 to alanine completely abolished cleavage at this site (Fig 1c) indicating that one (presumably the asparagine) or both of these residues is crucial for processing.

These results suggested that B cell lysosomes contain one or more novel cysteine endopeptidase(s) with possible specificity for asparagine. Although not previously described in antigen presenting cells, an enzyme with similar properties called legumain has been found in the seeds of legumimous plants[2,3], in *Schistosoma mansoni*[3] and recently in some mammalian tissues such as kidney and placenta[1]. We next partially

purified the TTCF processing activity from a crude B cell lysosome fraction to establish whether or not it was an asparaginyl endopeptidase. As shown in Figure 2b, a peak of TTCF processing activity eluted from a cation-exchange resin at 0.4M NaCl. This activity co-eluted precisely with an activity capable of cleaving the substrate Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide substrate (Fig 2a). Another protease activity, presumably cathepsin L and/or B, capable of cleaving the substrate Z-Phe-Arg-NHMec, partially overlapped the asparaginyl endopeptidase activity (Fig 2a). However this activity was completely inhibited by E64 (Fig 2a) while both the TTCF and Z-Ala-Ala-Asn-NHMec processing activity were unaffected (Fig 2b) confirming that a novel cysteine endopeptidase was involved. To compare this activity to the recently described mammalian form of legumain we generated anti-peptide antisera to several regions of the mammalian preprolegumain sequence[1]. Blotting of each fraction with an antisera to residues 125-140 (see methods) revealed the presence of a protein with the same mobility (35 kD) as purified pig kidney legumain which co-eluted with the TTCF and peptide substrate processing activity (Fig 2c). Finally, exactly the same processing sites were recognised by the crude lysosomal fraction, the partially purified TTCF processing activity and purified pig kidney legumain (Figs 1b & 2b, ref 1 and data not shown). Thus, mammalian legumain or a closely related enzyme, is present in human B lymphocytes and *in vitro*, is the major enzyme responsible for processing this antigen. We propose the name AEP (Asparaginyl endopeptidase) for this enzyme to distinguish it from the plant enzyme.

We decided to test the possibility that high concentrations of asparagine containing peptides might act as competitive inhibitors. As shown in Fig

3, the N and C-terminally blocked tetrapeptide Ala-Glu-Asn-Lys-NH (AENK) substantially inhibited the processing of TTCF by AEP (Fig 3a). Lys-Asn-Asn-Glu-NH (KNNE) also inhibited but was less effective. Importantly, the same concentrations of the glutamine analogues AEQK and KQQE did not inhibit TTCFr processing demonstrating specific competitive inhibition of AEP (Fig 3a). To establish that inhibition of AEP was specific, we measured the hydrolysis of different synthetic peptide substrates in the presence and absence of these tetrapeptides. As shown in Fig 3b, AENK completely blocked cleavage of Z-Val-Ala-Asn-NHMec by B cell lysosomal fractions but had no effect on the hydrolysis of the cathepsin L/B substrate, Z-Phe-Arg-NHMec or on the Cathepsin S substrate Z-Val-Val-Arg-HNMec.

If AEP plays a role in TTCF processing in antigen presenting cells then sufficiently high levels of the competing AENK peptide might be expected to interfere with normal class II MHC loading of TTCF epitopes. Following a 15 minute preincubation with or without tetrapeptides, freshly isolated peripheral blood mononuclear cells (PBMC) were incubated with TTCF antigen in the continued presence or absence of the peptides. At different times, the cells were washed, fixed and then co-cultured with different autologous T cell clones to assess expression of different peptide/MHC complexes. Presentation to most clones was detectable after 60 minutes of antigen pulsing in the absence of competing peptide but in the presence of AENK, presentation to some clones was either undetectable (AK 20 and AK 90; Fig 3b) or profoundly inhibited (eg AK 6). At later times there was some recovery of presentation of these epitopes as expected for a competitive inhibitor. Several controls ruled out the possibility that the inhibitory effect of the AENK competitor is

simply due to non-specific toxicity. First, presentation to other clones recognising other regions of TTCF was not affected by the AENK competitor (eg AK 111, 71 and 33: Fig 3c). Secondly, there was a clear differential sensitivity to AENK versus AEQK (Fig 3c), strongly suggesting that specific inhibition of AEP was responsible for the slowed kinetics of presentation. Thirdly, the inhibitory effects of AENK could be overridden by using TTCF pre-digested *in vitro* by AEP. In other words, the requirement for AEP *in vivo*, and hence the inhibitory effect of AENK, was by-passed by AEP cleavage *in vitro* (data not shown). Taken together our results reveal AEP as a new and highly specific processing activity in the class II MHC pathway. Preliminary studies show that AEP is present in a variety of other antigen presenting cell types.

Although we uncovered this processing activity using an antigen proteolysis assay, it is possible that it also plays a role in invariant chain processing as well. Splenocytes from cathepsin D gene targeted mice continued to produce invariant chain processing products even when all known cysteine protease activity were also suppressed by leupeptin[7]. Villadangos *et al* suggested that other non-cysteine proteases (besides cathepsin D) must be initiating Ii (invariant chain) cleavage. Cathepsin E is one possible candidate but so too is AEP since, although it is a cysteine protease, it is completely resistant to leupeptin (Fig 1a). Intact p31 Ii is readily cleaved by AEP *in vitro* and we are currently assessing the possibility that AENK also interferes with Ii processing. However, the differential effect of the AENK inhibitor on T cell clones and the fact that pre-digestion of antigen with AEP abolished the inhibitory effect of the AENK inhibitor argues that its primary inhibitory effect in our studies was on TTCF processing.

Can any special significance be attached to the existence of an asparaginyl endopeptidase in antigen presenting cells? One possibility is that microbial proteins might in general be better substrates than host proteins since the latter could resist AEP attack by N-glycosylation of particularly sensitive asparagine residues. It is very striking that while TTCF is an extremely good substrate for AEP, cleavage occurs at only 3 of its 47 asparagine residues. This suggests that there may be additional determinants of AEP specificity to be identified and that strategic N-glycosylation might give substantial protection. Thus the innate immune systems ability to bind microbial antigen through recognition of non-self molecular 'patterns', might be harnessed to an 'innate' bias towards microbial antigen processing as well. In addition, our data raise the possibility that disturbances of N-glycosylation might result in altered processing of self proteins by AEP leading to presentation of 'cryptic' epitopes with possible pathological consequences.

Methods

TTCF processing activity. Lysosomal fractions from the human B cell line EDR were prepared on 27% Percoll density gradients as previously described[8] using ^{125}I-labelled transferrin (internalised for 30 minutes at 37°C) and β-hexosaminidase to identify endosomal/plasma membrane and lysosomal fractions respectively. The peak of lysosomes was collected by centrifugation at 40,000 g for 1 hour, removed from the underlying Percoll pellet, collected again at 50,000 g for 1 hour and stored frozen. For separation of leupeptin-insensitive cysteine protease activity, 7.5×10^9 cells were homogenised in a ball-bearing homogenizer and nuclei and

unbroken cells removed by centrifugation at 2,000 g for 10 minutes. A membrane pellet was collected at 40,000 g and solubilised in 50 mM citrate buffer, pH 5.5 containing 0.1% CHAPS (3-([cholamidopropyl)dimethylammonio]-1-propane-sulphonate). The extract was centrifuged at 2,000 g for 20 minutes and then applied to a Mono-S column (Pharmacia[?]). Fractions were eluted with a gradient of NaCl and monitored for TTCF processing activity.

Proteins and peptides. A histidine tagged derivative of the C terminal domain of tetanus toxin was prepared[9,10] and purified[10] as described. This protein has residues 872-1315 of the complete toxin (1-1315) preceded by the sequence MGHGHHHHHHHHHHSSGHIEGRHI. Mutagenesis of residues 1184/5 was performed using template EH106 obtained by cloning the His-TTCF (Reference 10) as an XbaI/BamHI fragment into pSL1180 (Pharmacia). Site directed mutagenesis of residues 1184/85 was performed according to the method of Mikaelian and Sergent[11] using the mutagenic primer CGC TAC ACT CCG AAC GCG GCG ATC GAT TCT TTC GTT and flanking primers M13 rev (AGCGGATAACAATTTCACACAGGA) and M13 seq (GTAAAACGACGGCCAGT). After sequencing to confirm mutagenesis, recloned back into pET16B for expression. Tetrapeptides were synthesised using Fmoc chemistry leaving the C-terminus amidated and the N-terminus retaining the Fmoc group. LHVS was a kind gift from Hidde Ploegh, MIT.

Antigen presentation. Peripheral blood mononuclear cells were prepared by Ficoll/Paque centrifugation and used fresh. T cell clones specific for TTCF were established from donor A.K. according to[12]. Epitope

mapping was performed using a set of 88 peptides, 17 residues in length spanning the TTCF sequence (Chiron Mimotopes). Clones AK 6, 90, and 71 recognise the peptides 1235-1245, 1145-1156 and 950-961 respectively. Clones 111 and 33 recognise epitopes within the regions 1225-1276 and 1104-1153. The epitope recognised by clone 20 has not been mapped. Peptides AENK or AEQK were dissolved in water, made isotonic with NaCl and diluted into RPMI growth medium. T cell proliferation assays were performed essentially as described[12,13]. Briefly, following antigen pulsing (30 μg/ml TTCF) with or without tetrapeptides (1 mg/ml) PBMC were washed in PBS and fixed for 45 seconds in 0.05% glutaraldehyde. Glycine was added to a final concentration of 0.1M and the cells washed 5 times in RPMI 1640 medium containing 1% FCS before co-culture with T cell clones in round-bottom 96-well microtitre plates. After 48 hours the cultures were pulsed with 1 μCi of ^{3}H-thymidine and harvested 16 hours later.

References for Example

1. Chen, J.M., *et al* (1997) *J. Biol. Chem.* **272**, 8090-8.
2. Kembhavi, A.A., Buttle, D.J., Knight, C.G. & Barrett, A.J. (1993) *Arch. Biochem. Biophys.* **303**, 208-13.
3. Dalton, J.P., Hola Jamriska, L. & Brindley, P.J. (1995) *Parasitology* **111**, 575-80.
4. Riese, R.J., *et al* (1996) *Immunity* **4**, 357-66.
5. Watts, C. (1997) *Annu Rev Immunol* **15**, 821-50.
6. Fineschi, B. & Miller, J. (1997) *Trends Biochem Sci* **22**, 377-382.
7. Villadangos, J.A., Riese, R.J., Peters, C., Chapman, H.A. & Ploegh, H.L. (1997) *J. Exp. Med.* **186**, 549-60.
8. Davidson, H.W., West, M.A. & Watts, C. (1990) *J Immunol* **144**, 4101-9.
9. Makoff, A.J., Ballantine, S.P., Smallwood, A.E. & Fairweather, N.F. (1989) *Bio/Technology* 7, 1043-1046.
10. Hewitt, E.W., *et al* (1997) *J. Immunol.* **159**, 4693-9.
11. Mikelain, I. & Sergent, A. (1992) *Nucleic Acids Res.* **20**, 376.
12. Lanzavecchia, A. (1985) *Nature* **314**, 537-539.
13. Pond, L. & Watts, C. (1997) *J. Immunol.* **159**, 543-53.

References for synthetic methods desribed in Figure 4

1. Thomson, R.C. *Meth. Enzymol.* **46**, 220-

2. Vinitsky, A., Cardozo, C., Sepp-Lorenzino, L, Michaud, C. & Orlowski, M. Inhibition of the proteolytic activity of the multicatalytic proteinase complex by Substrate-related peptidyl aldehydes. *J. Biol. Chem.* **269**, 29860-29866 (1994).

3. Shaw, E. & Green, G.D.J. Inactivation of thiol proteases with peptidyl diazomethyl ketones. *Meth. Enzymol.* **80**, 820-826 (1981).

4. Shaw, E. Peptidyl diazomethanes as inhibitors of cysteine and serine proteases. *Meth. Enzymol.* **244**, 649-656 (1994).

5. Williams, E.B. & Mann, K.G. Peptide chloromethyl ketones as labelling reagents. *Meth. Enzymol.* **222**, 503-513 (1993).

6. Palmer, J.T., Rasnick, D., Klaus, J.L. & Bromme, D. Vinyl sulphones as mechanism-based cystein protease inhibitors. *J. Med. Chem.* **38**, 3193-96 (1995).

7. Krantz, A. Peptidyl (acyloxy) methanes as quiescent affinity labels for cysteine proteases. *Meth. Enzymol.* **244**, 656-671 (1994).

8. Bromme, D. & Memuth, H-U. N, O-diacyl hydroxamates as selective and irreversible inhibitors of cystein proteases. *Meth. Enzymol.* **244**, 671-685.

CLAIMS

1. A method of modulating the immune response in a patient in need of such modulation, the method comprising administering to the patient an effective amount of an inhibitor of asparaginyl endopeptidase.

2. A method according to Claim 1 wherein the patient has or is at risk of a disease which involves MHC Class II molecules.

3. A method according to Claim 1 or 2 wherein the disease is an autoimmune disease.

4. A method according to Claim 3 wherein the disease is rheumatoid arthritis.

5. A method according to any one of the preceding claims wherein the inhibitor is a competitive inhibitor.

6. A method according to Claim 5 wherein the competitive inhibitor is a peptide comprising is an asparagine-containing peptide.

7. A method according to Claim 6 wherein the peptide is an N and C-terminal blocked peptide Ala-Glu-Asn-Lys-NH (AENK) or Lys-Asn-Asn-Glu-NH (KNNE).

8. A method according to Claim 1 to 4 wherein the inhibitor is a non-competitive or irreversible inhibitor.

9. A method according to Claim 8 wherein the inhibitor has the structure Bl-$(X)_n$-Asn-Q where Bl is any suitable N terminal blocking group; X is an amino acid residue; n is between 1 and 100, Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine of asparaginyl endopeptidase.

10. A method of reducing the processing of a protein antigen by a MHC Class II molecule by a cell, the method comprising contacting the cell with an inhibitor of asparaginyl endopeptidase.

11. A method according to Claim 10 wherein the inhibitor is a competitive inhibitor.

12. A method according to Claim 11 wherein the competitive inhibitor is a peptide comprising an asparagine-containing peptide.

13. A method according to Claim 12 wherein the peptide is an N and C-terminal blocked peptide Ala-Glu-Asn-Lys-NH (AENK) or Lys-Asn-Asn-Glu-NH (KNNE).

14. A method according to Claim 10 wherein the inhibitor is a non-competitive or irreversible inhibitor.

15. A method according to Claim 14 wherein the inhibitor has the structure Bl-$(X)_n$-Asn-Q where Bl is any suitable N terminal blocking group; X is an amino acid residue; n is between 1 and 100, Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine of asparaginyl endopeptidase.

14. Use of an inhibitor of asparaginyl endopeptidase in the manufacture of a medicament for modulating the immune response in a patient in need of such modulation.

15. Use according to Claim 14 wherein the patient has or is at risk of a disease which involves MHC Class II molecules.

16. Use according to Claim 14 or 15 wherein the disease is an autoimmune disease.

17. Use according to Claim 16 wherein the disease is rheumatoid arthritis.

18. Use according to any one of Claims 14 to 17 wherein the inhibitor is a competitive inhibitor.

19. Use according to Claim 18 wherein the competitive inhibitor is a peptide comprising is an asparagine-containing peptide.

20. Use according to Claim 19 wherein the peptide is an N and C-terminal blocked peptide Ala-Glu-Asn-Lys-NH (AENK) or Lys-Asn-Asn-Glu-NH (KNNE).

21. Use according to any one of Claims 14 to 17 wherein the inhibitor is a non-competitive or irreversible inhibitor.

22. Use according to Claim 21 wherein the inhibitor has the structure Bl-$(X)_n$-Asn Q where Bl is any suitable N terminal blocking group; X is an amino acid residue; n is between 1 and 100, Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine of asparaginyl endopeptidase.

23. Use of an inhibitor of asparaginyl endopeptidase for modulating the immune response in a patient in need of such modulation.

24. Use of an inhibitor of asparaginyl endopeptidase for reducing the processing of a protein antigen by a MHC Class II molecule by a cell.

25. An inhibitor of asparaginyl endopeptidase for use in medicine.

26. A pharmaceutical composition comprising an inhibitor of asparaginyl endopeptidase and a pharmaceutically acceptable carrier.

27. A method of identifying a compound for modulating Class II MHC antigen processing the method comprising contacting a test compound with asparaginyl endopeptidase and selecting a compound which reduces its activity.

28. A method according to Claim 27 wherein the activity of asparaginyl endopeptidase is measured using a substrate which upon cleavage by said endopeptidase, yields a readily detectable product.

29. A method according to Claim 28 wherein the substrate is Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide and the product is fluorescent.

30. A method according to any one of Claims 27 to 29 the method further comprising the step of determining whether the so selected compound is capable of substantially inhibiting the loading and presentation of peptides on an appropriate Class II MHC molecule-containing cell.

31. A method according to Claim 30 wherein it is determined whether the so selected compound is capable of substantially inhibiting T cell activation by an appropriate Class II MHC molecule-containing cell.

32. A non-human transgenic animal wherein a gene encoding asparaginyl endopeptidase has been modified and the animal expresses substantially no asparaginyl endopeptidase from said gene.

33. A non-human transgenic animal according to Claim 32 which is a mouse.

34. A non-human transgenic animal according to Claim 32 or 33 further comprising a genetic background which predisposes to an autoimmune disease either spontaneously or upon administration of protein antigen.

35. A non-human transgenic animal according to Claims 32 or 33 further transgenic for a human Class II MHC molecule and, optionally, further transgenic for human CD4.

Abstract

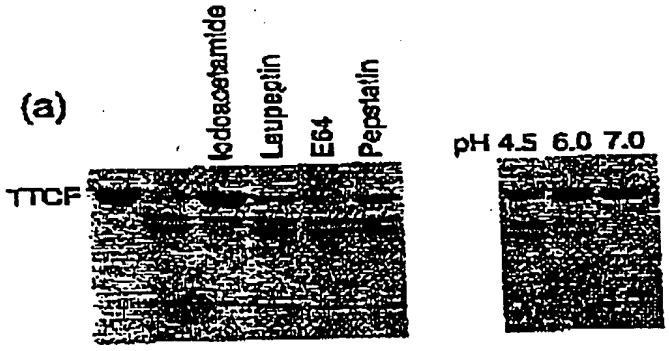
A method of modulating the immune response in a patient in need of such modulation, the method comprising administering to the patient an effective amount of an inhibitor of asparaginyl endopeptidase.

A method of reducing the processing of a protein antigen by a MHC Class II molecule by a cell, the method comprising contacting the cell with an inhibitor of asparaginyl endopeptidase.

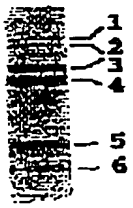
FIG. 1

Fig 1

Iodoacetamide
Leupeptin
E64
Pepstatin



(b)



Fragment	Sequence/ cleavage site
2,4	RHIDN EEDID
5	YTPNN EIDSF
6	GNAFN NLDRI

RHIDN EEDID
YTPNN EIDSF
GNAFN NLDRI

Fig 1c

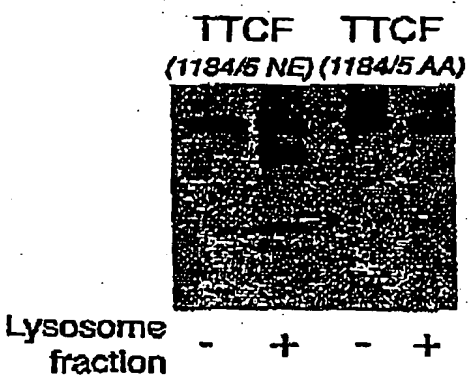


Fig 2

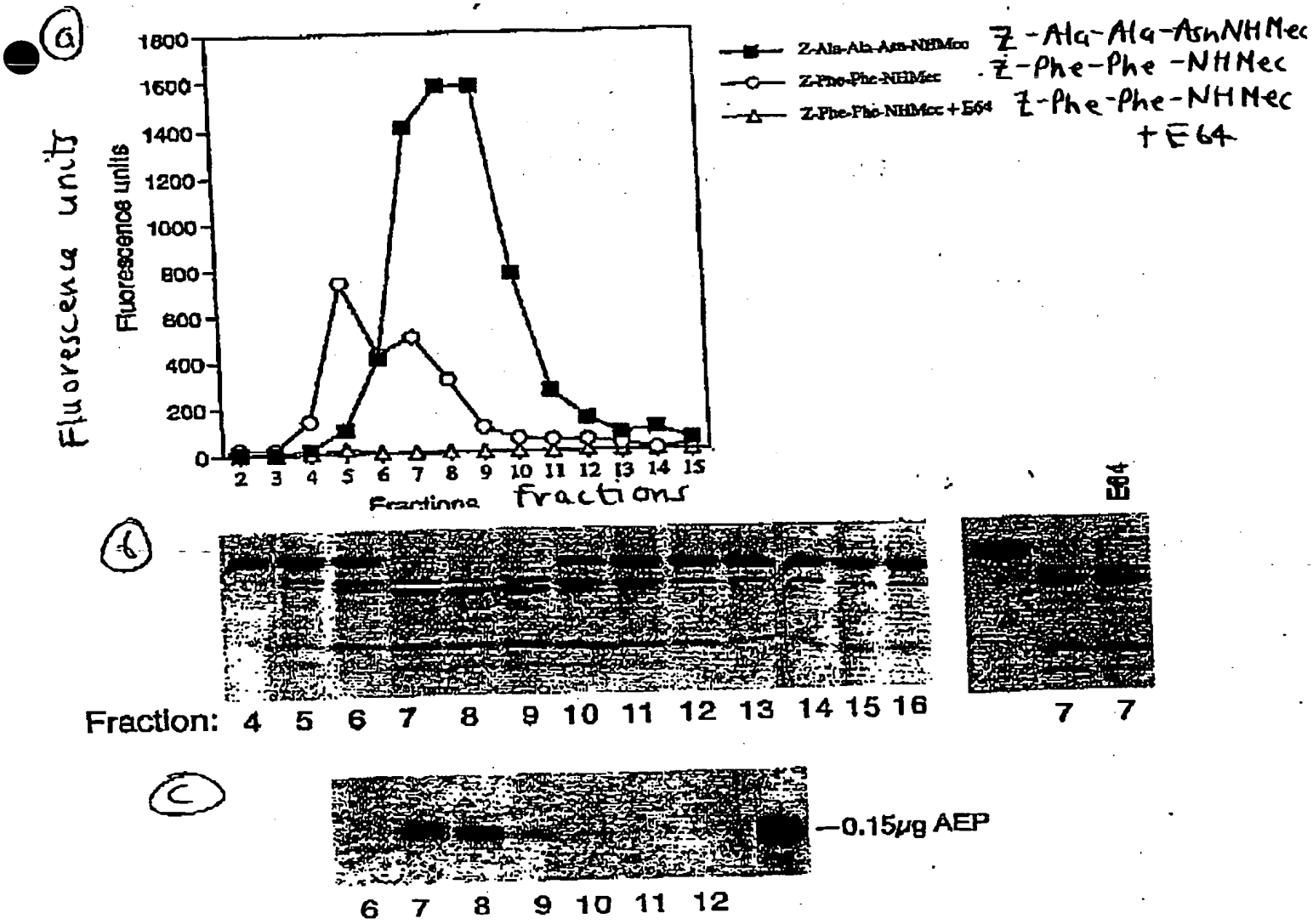

a
Fluorescence units
Fluorescence units
Fractions
Z-Ala-Ala-Asn-NHMec
Z-Phe-Phe-NHMec
Z-Phe-Phe-NHMec+E64
Z-Ala-Ala-AsnNHMec
Z-Phe-Phe-NHMec
Z-Phe-Phe-NHMec +E64
b
E64
Fraction: 4 5 6 7 8 9 10 11 12 13 14 15 16
7 7
c
—0.15μg AEP
6 7 8 9 10 11 12

Fig 3a

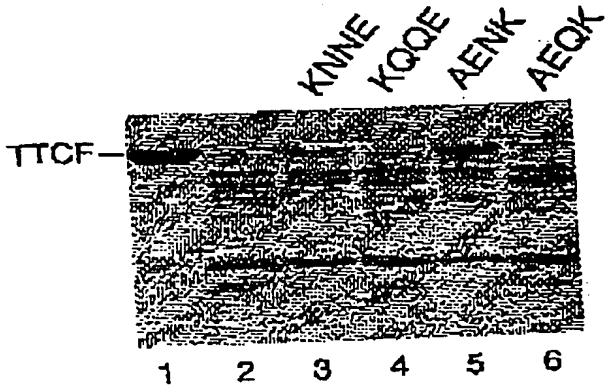
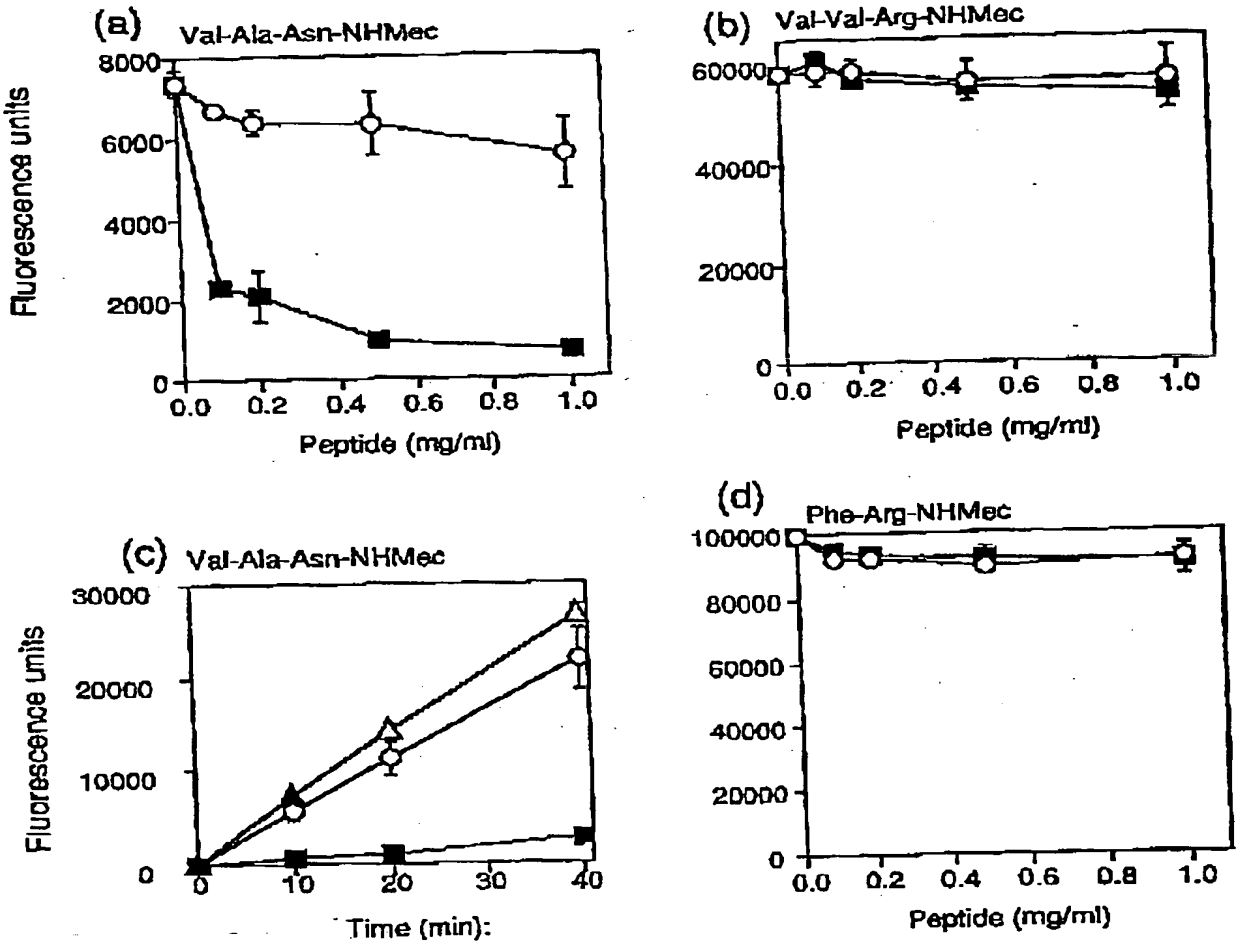


Fig 36


(a) Val-Ala-Asn-NHMec
Fluorescence units
8000
6000
4000
2000
0
0.0 0.2 0.4 0.6 0.8 1.0
Peptide (mg/ml)
(b) Val-Val-Arg-NHMec
60000
40000
20000
0
0.0 0.2 0.4 0.6 0.8 1.0
Peptide (mg/ml)
(c) Val-Ala-Asn-NHMec
Fluorescence units
30000
20000
10000
0
0 10 20 30 40
Time (min):
(d) Phe-Arg-NHMec
100000
80000
60000
40000
20000
0
0.0 0.2 0.4 0.6 0.8 1.0
Peptide (mg/ml)

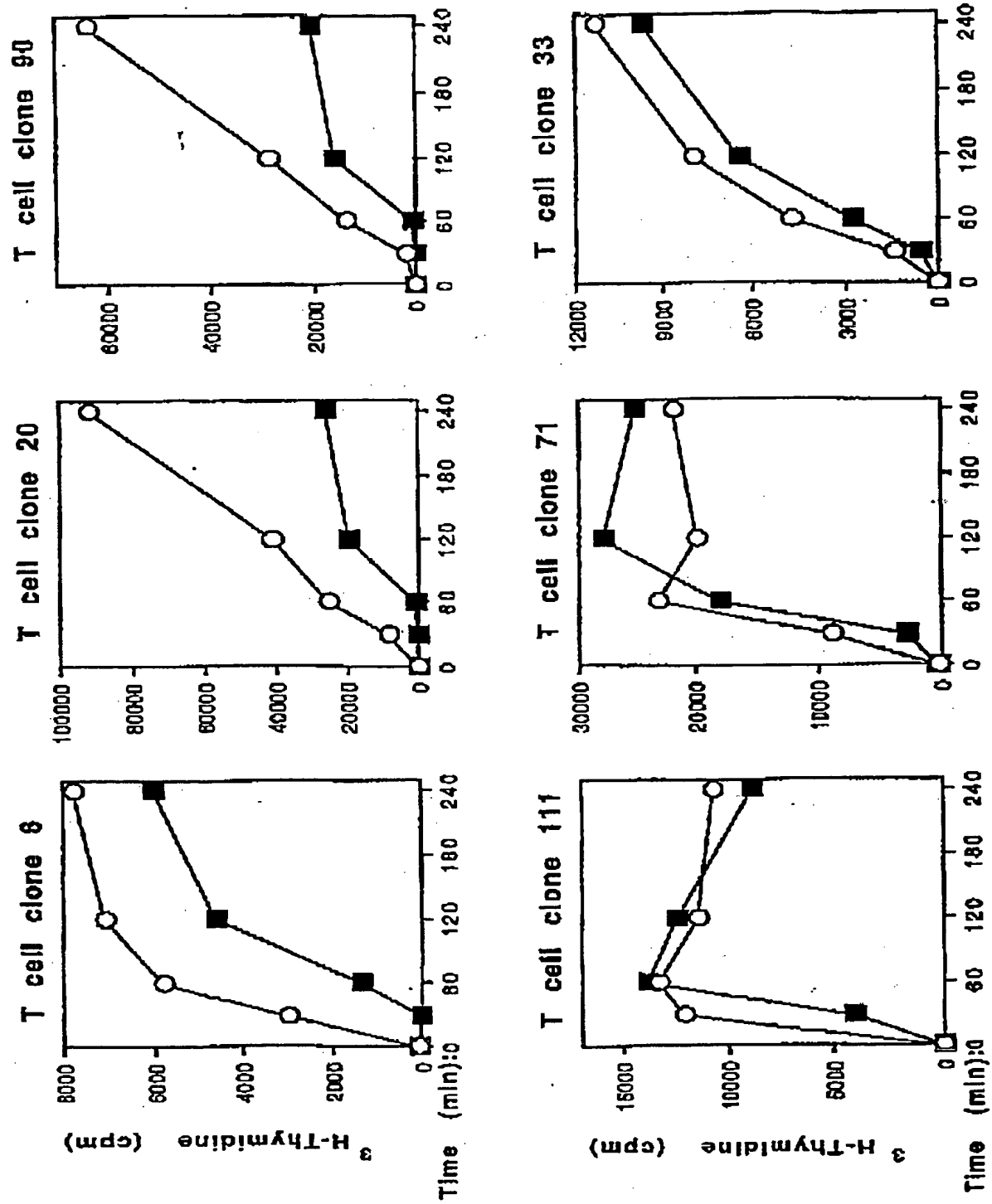

T cell clone 90
T cell clone 33
T cell clone 20
T cell clone 71
T cell clone 8
T cell clone 111
^{3}H-Thymidine (cpm)
Time (min)
0
60
120
180
240

Fig 3c

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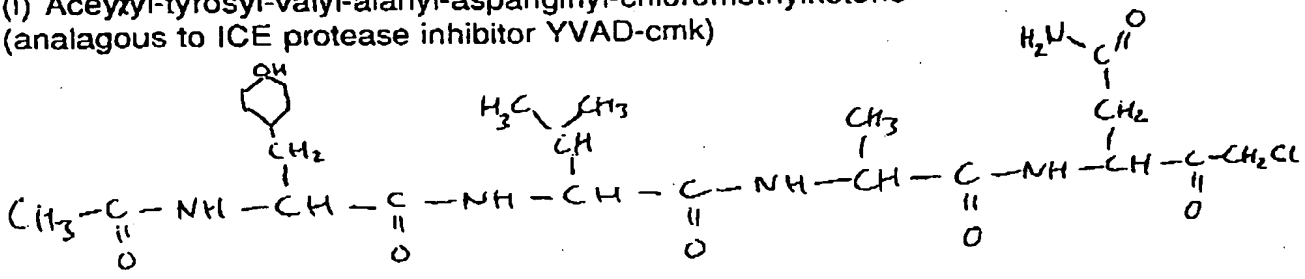
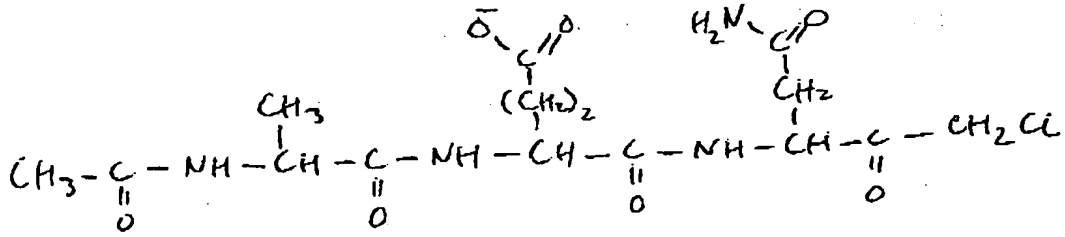
Figure 4 (page 1 of 6)

Peptide chloromethylketones (ref 5)

(i) Acetyl-tyrosyl-valyl-alanyl-aspariginyl-chloromethylketone
(analagous to ICE protease inhibitor YVAD-cmk)



(ii) Acetyl-alanyl-glutamyl-aspariginyl-chloromethylketone



(iii) Acetyl (or benzyloxycarbonyl)-$(X)_n$-Asparaginyl-chloromethylketone

Where X = any amino acid

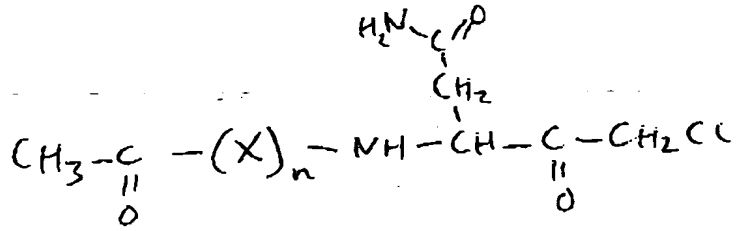
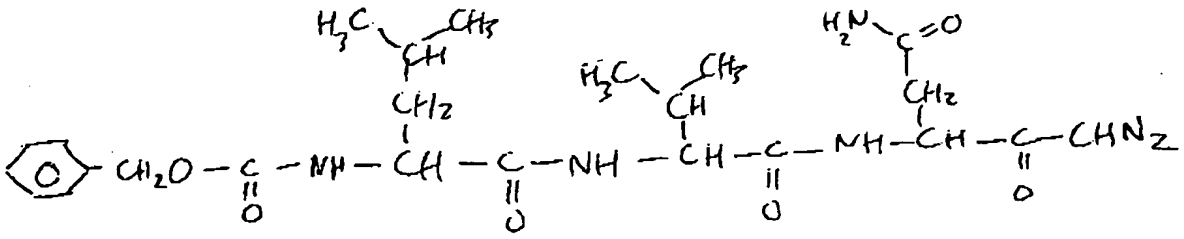


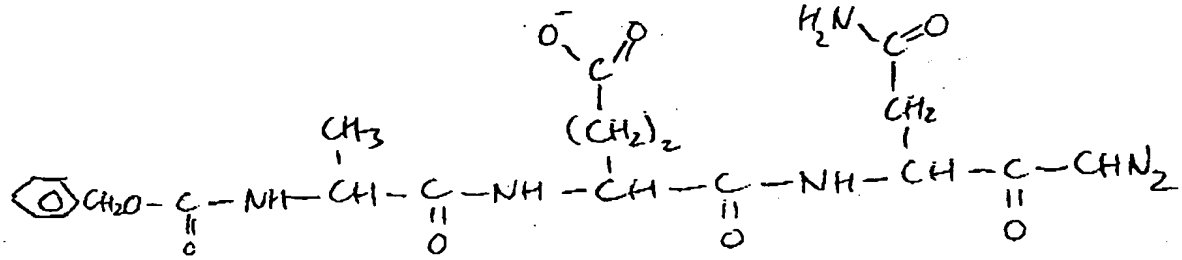
Figure 4 (page 2 of 6)

Peptidyl diazomethanes (refs 3,4) (have the general structure: R-C(=O)CHN_2

(i) benzyloxycarbonyl-leucyl-valyl-asparaginyl-diazomethane



(ii) alanyl-glutamyl-asparaginyl diazomethane



(iii) Z-$(X)_n$-asparaginyl-diazomethane

Where Bl= acetyl or benzyloxycarbonyl and X= any amino acid

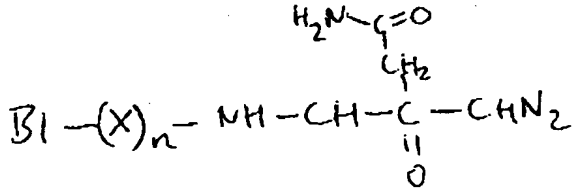


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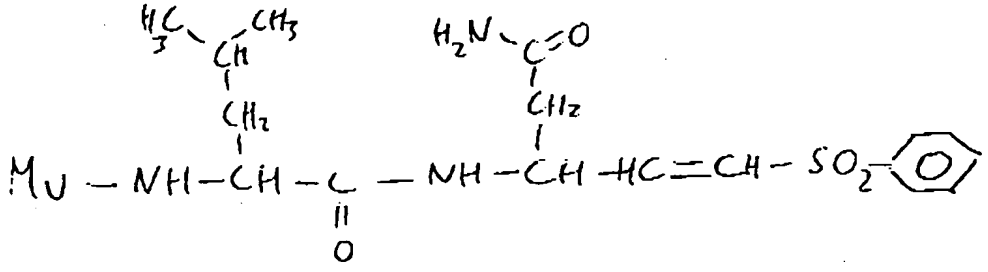
Figure 4 (page 3 of 6)

Peptide vinyl sulphones (ref 6)

(i) Morpholinurea-leucyl-asparaginyl-vinylsulphone-phenyl

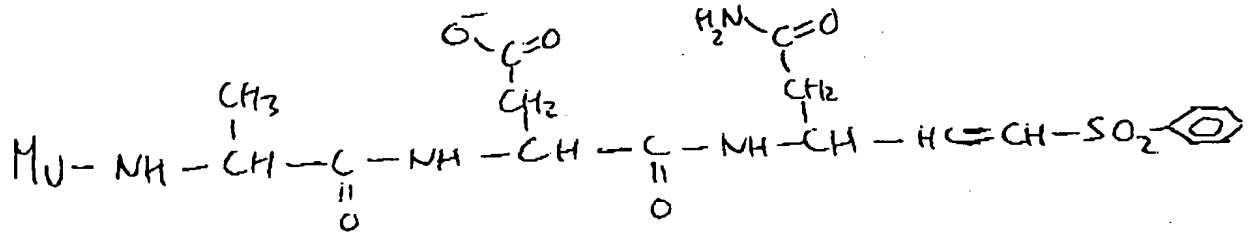
(Acetyl or benzyloxycarbonyl can replace morpholinurea)



MU = morpholinurea

(ii) Morpholinurea-alanyl-glutamyl-asparaginyl-vinylsulphone-phenyl

(Acetyl or benzyloxycarbonyl can replace morpholinurea)



(iii) Bl-$(X)_n$-asparaginyl -vinylsulphone-R

Where Bl= N-terminal blocking group (acetyl, morpholinurea or benzyloxycarbonyl, X= any amino acid and R=alkyl or aryl terminating group.

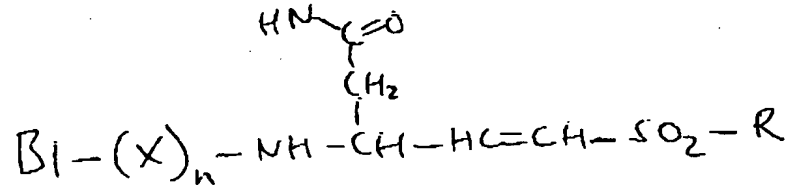
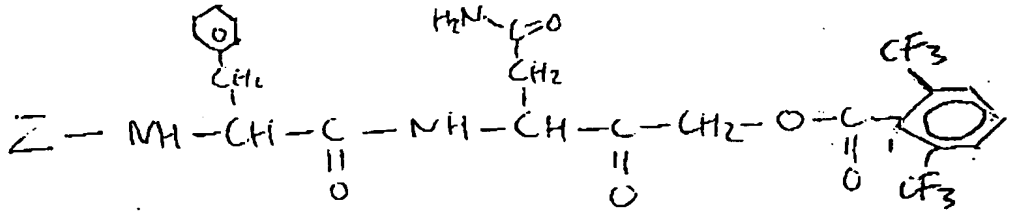


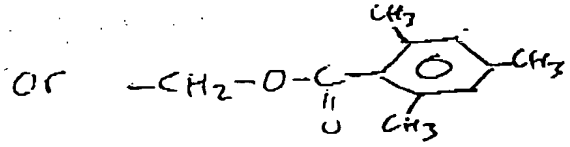
Figure 4 (page 4 of 6)

Peptidyl (acyloxy) methanes (ref 7)

(i) Z-Phenyalanyl-asparaginyl-CH_2OCO-[2,6-$(CF_3)_2$Phenyl

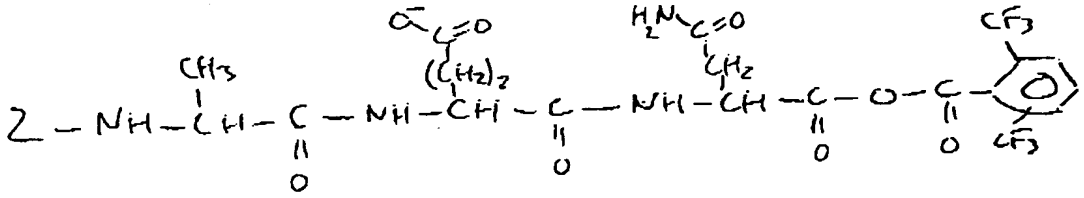
(ii) Z-Phenyalanyl-asparaginyl-CH_2OCO-[2,4,6-(CH_3) Phenyl

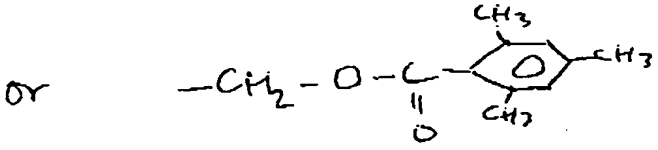




(ii) Z-alanyl-glutamyl-asparaginyl-CH_2OCO-[2,6-$(CF_3)_2$Phenyl

(iv) Z-alanyl-glutamyl-asparaginyl-CH_2OCO-[2,4,6-(CH_3) Phenyl





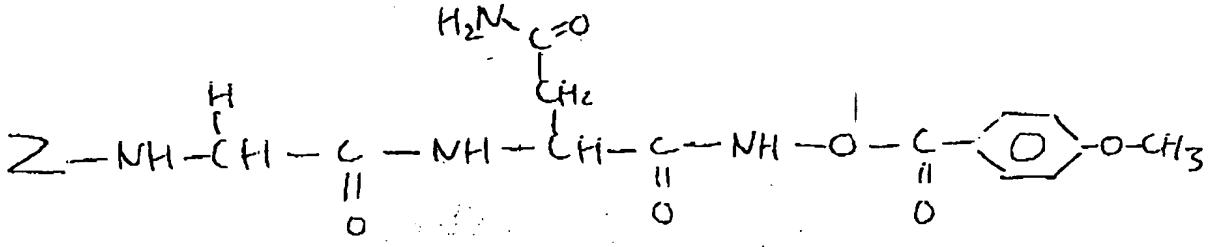
(iv) Z-(X)n-asparaginyl- CH_2OCO-R

Where X=any amino acid and R=[2,6-$(CF_3)_2$Phenyl or [2,4,6-(CH_3) Phenyl or other acyloxy methane group

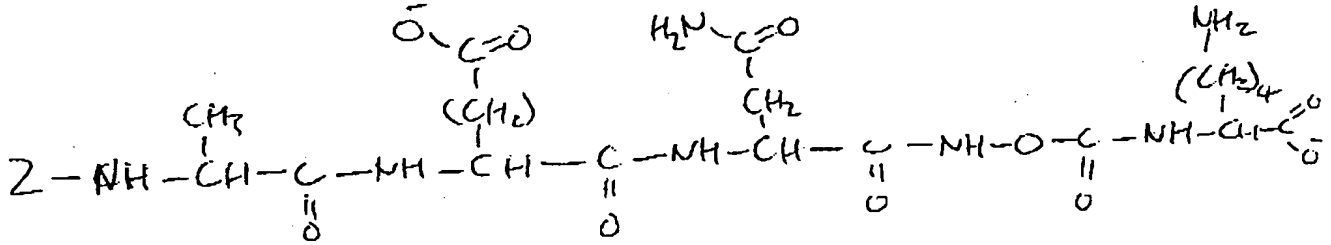
Figure 4 (page 5 of)

N,O-diacyl hydroxamates (ref 8)

(i) Z-Glycyl-asparaginyl-NHO-benzoyl(4-OCH3)

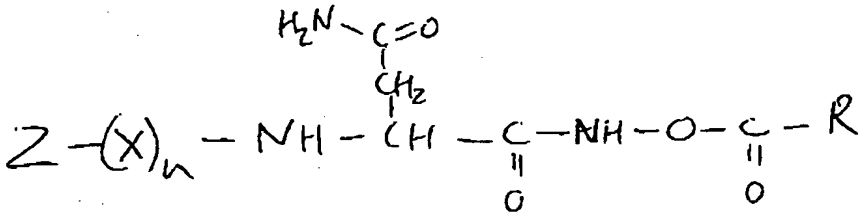


(ii) Z-alanyl-glutamyl-asparaginyl-NHO-CO-lysine-NH



(iii) Z-(X)n-asparaginyl-NHO-CO-R

Where Z= benzyloxycarbonyl or other blocking group, X= any amino acid, and R= any O-acyl group.



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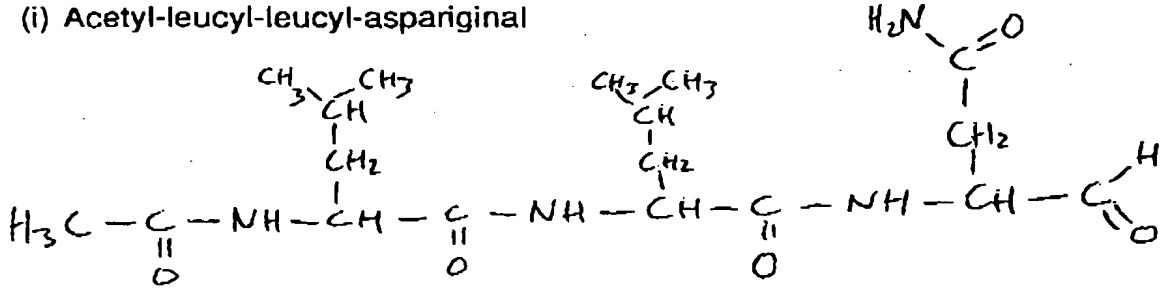
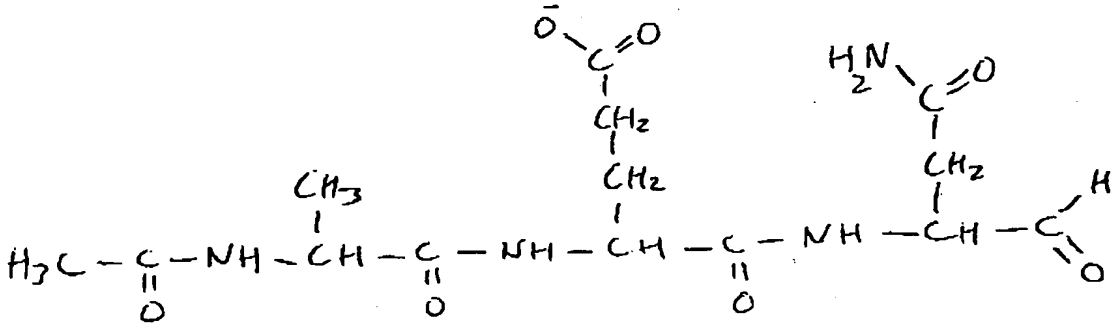
Peptide aldehydes (refs 1 & 2) :

(i) Acetyl-leucyl-leucyl-aspariginal



(ii) Acetyl-alanyl-glutamyl-aspariginal



(iii) Acetyl (or other blocking group)-$(X)_n$-Asparaginal

—where X denotes any amino acid(s) in peptide linkage.

Elastatinal also blocks AEP. A more specific variant would be:

(iv)

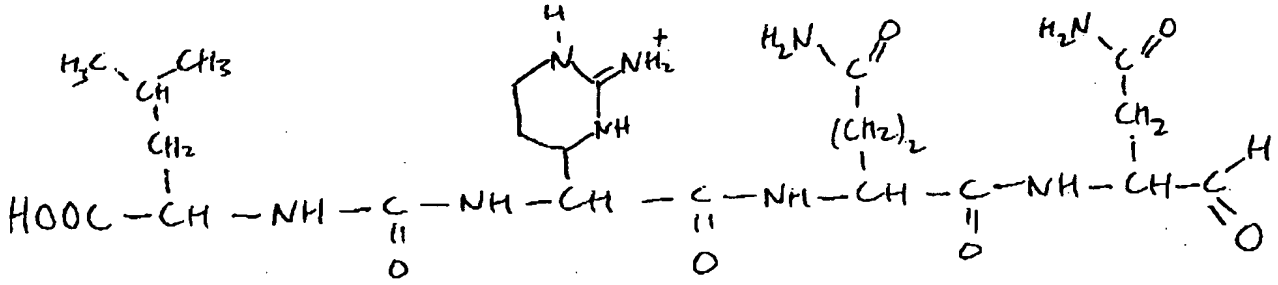
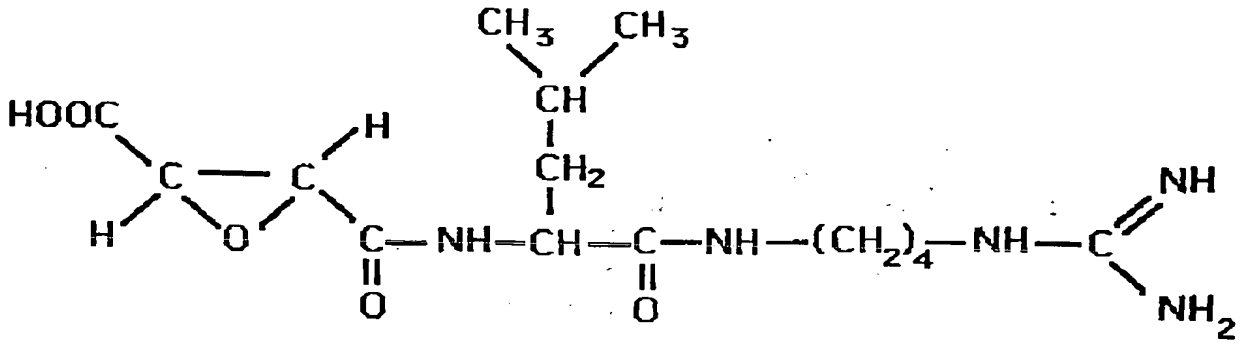


Figure 5 (page 1 of 6)

E-64

Structure: L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane or N-[N-(L-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine



Inhibition spectrum: Irreversible inhibitor of cysteine proteinases. Does not inhibit serine proteinases.

Mechanism of action: Forms a thioether bond with the thiol of the active cysteine (1:1 enzyme/inhibitor molar ratio)

Properties: E-64 is soluble in aqueous solutions (stock solution 1 mM). Stable from pH 2.0 to pH 10.0 but unstable in ammonia or strong acids, which destroy the epoxide ring. Excellent active-site titrant of cysteine proteinases. Originally isolated from the culture medium of a sold mold, *Aspergillus japonicus* .
MW: 357.4

Suggested final concentration: 1-10 µM

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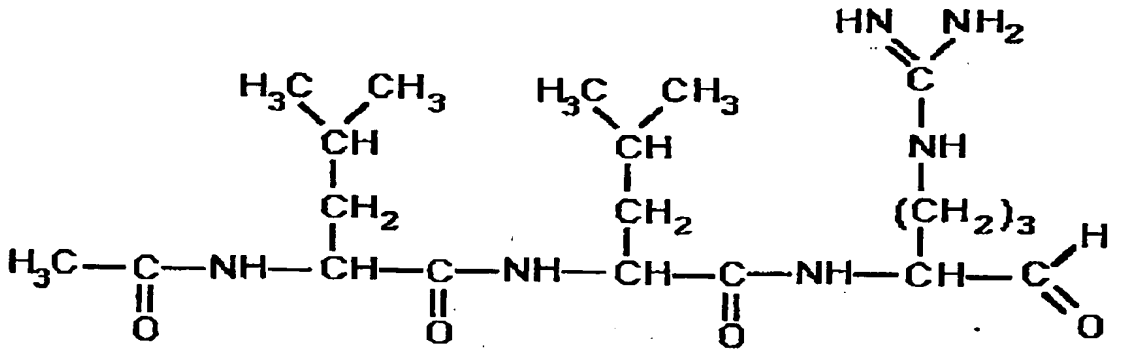
Figure 5 (page 2 of 6)

Leupeptin

Structure: Acetyl-leucyl-leucyl-arginal



Inhibition spectrum: inhibits serine (trypsin (Ki=13 μM), plasmin, porcine kallikrein) and cysteine proteinases (papain, cathepsin B). Does not inhibit chymotrypsin and thrombin.

Mechanism of action: Competitive and reversible inhibitor. Inhbition may be relieved by an excess of substrate.

Properties: Soluble in water, ethanol, acetic acid and DMF (Stock solution: 10 mM)
MW: leupeptin: 426.6; leupeptin hemisulphate monohydrate: 542.7

Suggested final concentration: 1-10 μM (0.5-1 μg/ml)

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Figure 5 (page 3 of 6)

Antipain

Structure: [(S)-1-Carboxy-2-Phenyl]-carbamoyl-Arg-Val-arginal

Inhibition spectrum: Inhibits papain, trypsin and plasmin to a lesser extent. More specific for papain and trypsin than leupeptin. The inhibitory potency of antipain is 100-fold higher than that of elastatinal.

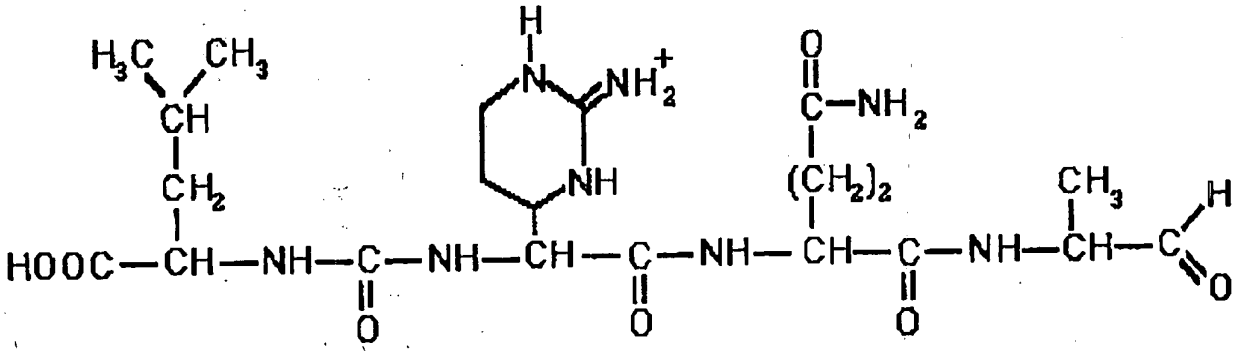
Mechanism of action: Formation of a hemicetial adduct between the aldehyde group of the inhibitor and the active serine of the proteinase.

Properties: Soluble in H_2O, methanol and DMSO (Stock solution: 10 mM). Stable at -20° C.
MW: 604.7 for antipain; 677.6 for antipain dihydrochloride

Figure 5 (page 4 of 6)

Elastatinal

Structure: Leu-(Cap)-Gln-Ala-al,
N-[(S)-1carboxy-isopentyl)-carbamoyl-alpha-(2-iminohexahydro-4(S)-pyrimidyl]-
L-glycyl-L-glutaminyl-L-alaninal



Inhibition spectrum: Inhibits elastase-like serine proteinases (Ki= 0.24 µM for porcine pancreatic elastase, Ki=50-80 µM for human leukocyte elastase). Specific for elastases, does not inhibit chymotrypsin or trypsin.

Mechanism of action: Formation of a hemicetal adduct between the aldehyde group of the inhibitor and the active serine of the proteinase.

Properties: Soluble in water (Stock solution: 10 mM)
MW: 512.6

Suggested final concentration: 10-100 µM

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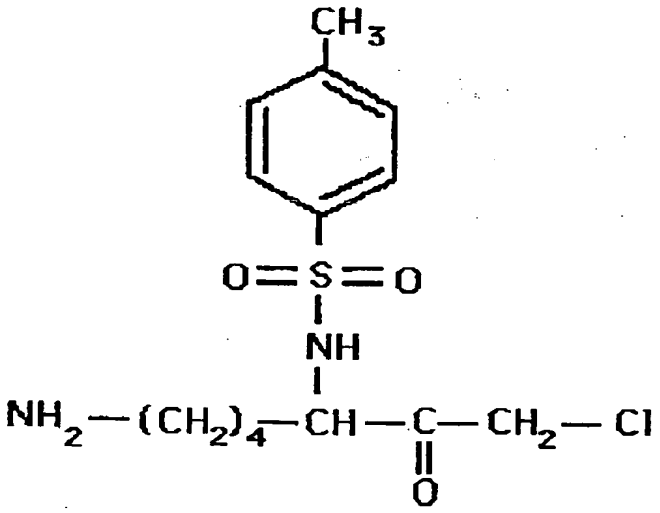
Figure 5 (page 5 of 6)

TLCK

Structure: Tosyl Lysyl ChloromethylKetone:
1-Chloro-3-tosylamido-7-amino-2-heptanone



Inhibition spectrum: Irreversible inhibitor of trypsin. Also inhibits some serine proteases (kallikrein, thrombin, plasmin) and some cysteine proteases such as papain, bromelain or ficin. Does not inhibit chymotrypsin and zymogens.

Mechanism of action: Requires an active enzyme and forms a covalent bond with His of the active site. Does not react with zymogens so addition of TLCK in a crude extract inactivates only active protease. Does not react with protease-inhibitor complexes.

Properties: TLCK salts are soluble in water up to 20 mg/ml. More stable at pH less or equal to 6.0.
Soluble up to 5 mg/ml in methanol or to 1 mg/ml in NaCl 0.15 M.
MW: 369.3 (TLCK.HCl)

Suggested final concentration: 50 µg/ml

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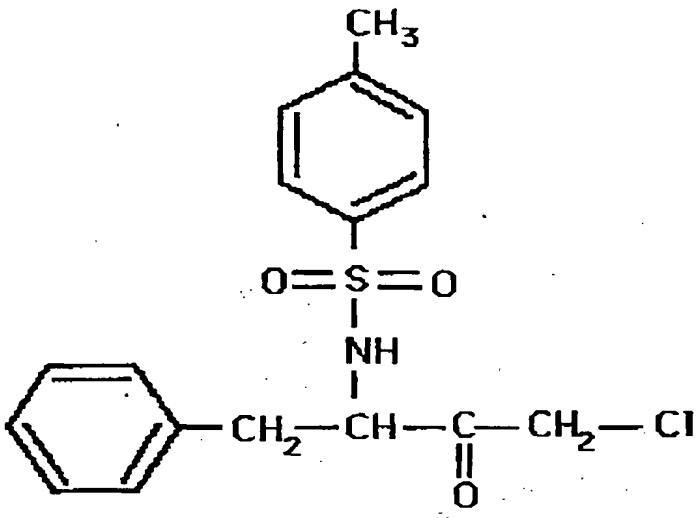
TPCK

Structure: Tosyl Phenylalanyl ChloromethylKetone:
1-Chloro-3-tosylamido-4-phenyl-2-butanone



Inhibition spectrum: Irreversible inhibitor of chymotrypsin. Also inhibits some proteases cleaving substrates with a Phe at position P1 and some cysteine proteases such as papain, bromelain or ficin. Does not inhibit trypsin and zymogens.

Mechanism of action: Requires an active enzyme and forms a covalent bond with His of the active site. Does not react with zymogens so addition of TPCK in a crude extract inactivates only active protease. Does not react with protease-inhibitor complexes.

Properties: TPCK is soluble in ethanol up to 20 mg/ml but poorly in water. It is more stable at slightly acidic or neutral pH. It is destroyed in less than 30 min. at pH 9.0.
MW: 351.9

Suggested final concentration: 100 µg/ml

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Eric Potter clarkson.